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SPECIFICATION

CROSSLINKED POLYSACCHARIDE MICROPARTICLES AND
METHOD FOR THEIR PREPARATIONTECHNICAL FIELD

5 [0001] The present invention relates to sustained-release drug carriers composed of crosslinked polysaccharide microparticles which allow sustained release of drugs, more particularly pharmacologically active proteins or peptides, as well as a method for their
10 preparation.

BACKGROUND ART

[0002] In recent years, an increasing number of formulations of pharmacologically active proteins or peptides have been developed for commercial use. However, such drugs usually have a short half-life in the blood and most of them are injections that must be administered repeatedly at frequent intervals, thus imposing excessive burdens on patients during drug administration. Hence, there is a demand for practical, sustained-release formulations of protein or peptide drugs, which exert their efficacy in as small amounts as possible and which permit reduced frequency of administration.
20

[0003] Sustained-release formulations of pharmacologically active proteins or peptides will cause denaturation or aggregation of the proteins or peptides during formulation preparation or sustained release, which results in a reduced recovery rate and constitutes a major obstacle to their development for commercial use. It has
25

been attempted to prepare sustained-release formulations based on a biodegradable polymer matrix such as polylactic acid-polyglycolic acid copolymer (PLGA), but such formulations have been reported to cause protein 5 denaturation and/or aggregation due to the hydrophobicity of the matrix, a drying step and/or a decrease in pH (see Non-patent Documents 1 and 2). On the other hand, there are also reports of sustained-release formulations based on a hydrophilic hydrogel matrix with reduced risks of these 10 problems, but such formulations are not ready for commercial use. In terms of safety, a material used as a sustained-release matrix should combine non-antigenicity, non-mutagenicity, non-toxicity and biodegradability. Thus, no sustained-release formulation is now ready for 15 commercial use in all aspects, i.e., encapsulation efficiency and recovery rate of proteins or peptides, as well as safety.

[0004] Some recent reports have proposed the use of polysaccharides as matrixes for drug carriers. Among them, 20 hyaluronic acid (HA), a biomaterial (polysaccharide) isolated from the vitreous body of bovine eyes in 1934 by K. Meyer, has been known as a major component of extracellular matrix for a long time. HA is a kind of glycosaminoglycan composed of disaccharide units in which D-glucuronic acid 25 and N-acetylglucosamine are linked to one another via $\beta(1 \rightarrow 3)$ glycosidic linkages.

[0005] There is no difference among species in the chemical and physical structure of HA and humans also have

a metabolic system for HA; HA is therefore one of the safest medical biomaterials in terms of immunity and toxicity. Recent years have enabled microbial mass production of high-molecular-weight HA and also have
5 allowed commercial use of HA in the fields of therapeutic agents for degenerated cartilage, cosmetics, etc.

[0006] There are also many reports of crosslinking techniques for HA as a matrix and sustained release formulations of protein or peptide drugs from HA gels.
10 Techniques known for gelling HA via chemical crosslinking include the carbodiimide method (see Patent Document 1), the divinylsulfone method (see Patent Document 2), and the glycidyl ether method (see Patent Document 3). In general, when a protein or peptide is introduced into a crosslinked
15 gel for encapsulation purposes, it results in a low introduction efficiency because of problems arising from compatibility and electrostatic repulsion between HA and the protein or peptide. In contrast, when *in situ* crosslinking is performed in the presence of a protein or
20 peptide, it is advantageous in that the protein or peptide can be held in a gel at a high encapsulation efficiency. There are some reports showing that such *in situ* crosslinking is adapted for encapsulation of proteins or peptides into HA gels to give sustained-release
25 formulations (see, e.g., Patent Document 4). However, there arises a problem of recovery rate when such an approach is used for *in situ* crosslinking of HA in the presence of proteins or peptides to prepare sustained-

release formulations. As an example, a method is reported in which a HA derivative (HA-HZ) modified to have a hydrazide group (HZ) is crosslinked with a crosslinking agent comprising N-hydroxysuccinimide (NHS) (see Patent Document 5). This method is intended for *in situ* crosslinking under physiological conditions and limits crosslinkage formation at pH 7.4 to pH 8.5. However, the inventors' investigations have confirmed that this method also results in low recovery rates of proteins or peptides from the thus obtained HA gel. This is because the proteins or peptides will be partially reacted (mainly at their amino groups) with the crosslinking agent during crosslinking reaction to give crosslinked proteins. This method also suffers from a problem in that denatured proteins or peptides remaining in the gel have reduced biological activity and, if anything, are responsible for the cause of antigenicity. Although it is an essential requirement for pharmaceutical preparations that the encapsulated drug is released at a high recovery rate, no method is known for chemically crosslinking and gelling HA without causing proteins or peptides to react. Also, another method has been reported to encapsulate proteins or peptides at high recovery rates, in which polyethylene glycol (PEG) is used as a matrix and crosslinked through nucleophilic addition reaction of unsaturated functional groups (see Patent Document 6), but this method suffers from a problem in that fragments of non-biodegradable PEG remain unabsorbed.

[0007] In actual fact, to formulate such sustained-release materials into injectable formulations, these materials should be formulated in the form of microparticles. Spray dryers are widely used in such attempts and are also reported to be used in formulating insulin (see Non-patent Documents 3 and 4) and rh anti-IgE antibody (see Non-patent Document 5) in the form of microparticles, as well as in encapsulated drugs into hyaluronic acid microparticles (see Patent Documents 7 and 8). However, since the microparticles thus obtained will be dissolved in a short time in the subcutaneous tissues, they have a very short period of sustained release and are less practical for sustained release purposes. There is another report in which chitosan is crosslinked during spray drying so as to encapsulate low-molecular drugs therein (see Non-patent Document 6). However, the release period is as short as a few minutes in this case, and aldehyde used as a crosslinking agent is highly reactive with a functional group such as an amino group and hence cannot be used for proteins, peptides and other low-molecular drugs having a functional group(s) such as an amino group.

Patent Document 1: International Publication No. WO94/02517

Patent Document 2: JP 61-138601 A

25 Patent Document 3: JP 5-140201 A

Patent Document 4: United States Patent No. 5827937

Patent Document 5: International Publication No. WO95/15168

Patent Document 6: International Publication No. WO00/44808

Patent Document 7: Japanese Patent No. 3445283

Patent Document 8: International Publication No. WO96/18647

Non-patent Document 1: J. Pharm. Sci., vol. 88, pp. 166-173,
1999

5 Non-patent Document 2: J. Microencapsulation, vol. 15,
pp. 699-713, 1998

Non-patent Document 3: Int. J. Pharm. 233, 227-237, 2002

Non-patent Document 4: J. Control. Rel. 91, 385-394, 2003

Non-patent Document 5: Biotech. and Bioeng. 60, 301-309,

10 1998

Non-patent Document 6: Int. J. Pharm. 187, 53-65, 1999

DISCLOSURE OF THE INVENTION

PROBLEMS TO BE SOLVED BY THE INVENTION

[0008] As stated above, no method is known for
15 preparation of injectable and biodegradable gel
microparticles, which satisfies a high encapsulation
efficiency, a high recovery rate and safety through *in situ*
chemical crosslinking, drying, microparticle formation and
drug encapsulation while retaining the biological activity
20 of drugs such as proteins or peptides. Moreover, no
example is known for long-acting sustained-release
formulations of drugs such as proteins or peptides, which
are prepared using such a method.

MEANS FOR SOLVING THE PROBLEMS

25 [0009] As a result of extensive and intensive efforts
made to overcome the problems stated above, the inventors
of the present invention have found that drugs such as
proteins or peptides can be efficiently encapsulated while

retaining their biological activity when a solution of a drug and a hyaluronic acid derivative having a crosslinkable functional group(s) is concentrated starting from a dilute state, where crosslinking proceeds slowly, to 5 reach a concentration which facilitates crosslinking, to thereby cause crosslinking reaction between HA molecules during concentration, so that the drug is encapsulated into the crosslinked hyaluronic acid. The inventors have also found that the crosslinked hyaluronic acid microparticles 10 thus obtained are injectable and most suitable as biodegradable and safe microparticle carriers for long-term sustained release for the purpose of encapsulating drugs such as proteins or peptides. These findings led to the completion of the present invention.

15 [0010] Namely, the present invention relates to injectable sustained-release formulations of drugs such as proteins or peptides encapsulated in gels, which are obtained through *in situ* crosslinking, microparticle formation and drying while retaining the biological 20 activity of the drugs such as proteins or peptides, as well as a method for their preparation.

[0011] Namely, in one aspect, the present invention provides a method for preparing crosslinked polysaccharide microparticles, which comprises the following steps:

- 25 a) preparing a dilute solution containing a polysaccharide derivative having a crosslinkable functional group(s);
b) dispersing the solution to form microparticulate

droplets; and

c) concentrating the solution contained in the droplets to facilitate crosslinking reaction of the polysaccharide derivative. In another aspect, the present invention 5 further provides such a method wherein step b) is a step in which the solution is dispersed by spraying to form microparticulate droplets.

[0012] In yet another aspect, the present invention also provides crosslinked polysaccharide microparticles 10 which can be prepared by the above method.

[0013] The present invention will be further described in more detail below.

[0014] The polysaccharide derivative used in the present invention is not limited in any way as long as it 15 is a polysaccharide derivative having a crosslinkable functional group(s). Among derivatives of glycosaminoglycans (acidic mucopolysaccharides including hyaluronic acid, chondroitin, chondroitin 4-sulfate, chondroitin 6-sulfate, dermatan sulfate, heparin, heparan 20 sulfate, keratan sulfate, etc.), preferred are those having a crosslinkable functional group(s), and particularly preferred are hyaluronic acid derivatives having a crosslinkable functional group(s).

[0015] Thus, in yet another aspect, the present 25 invention also provides a method for preparing crosslinked hyaluronic acid microparticles, which comprises the following steps:

a) preparing a dilute solution containing a hyaluronic

acid derivative having a crosslinkable functional group(s);

b) dispersing the solution to form microparticulate droplets; and

c) concentrating the solution contained in the droplets

5 to facilitate crosslinking reaction of the hyaluronic acid derivative. In another aspect, the present invention further provides such a method wherein step b) is a step in which the solution is dispersed by spraying to form microparticulate droplets. In yet another aspect, the 10 present invention also provides crosslinked hyaluronic acid microparticles which can be prepared by the above method.

[0016] The dilute solution found in step a) of the present invention is a solution containing substances such as a substrate and a reagent required for crosslinking 15 reaction. However, the dilute solution is highly diluted with a solvent and hence the reaction does not proceed or proceeds very slowly in this solution. Although its concentration is not limited in any way, it is for example 0.1% to 5%, and particularly 0.2% to 3%. The solvent used 20 in the present invention may be any solvent commonly used in the art or a mixture thereof and includes, without any limitation, water, DMSO, ethanol, N-methylpyrrolidone, and supercritical carbon dioxide fluid.

[0017] In step b) of the present invention, the dilute 25 solution may be dispersed in the form of microparticulate droplets by using any technique commonly used in the art. Examples include, without any limitation, techniques for spraying the dilute solution and techniques to form an

emulsion by mixing the dilute solution with another fluid. The microparticulate droplets thus obtained may have an average particle diameter of, for example, 0.04 μm to 1.5 mm, preferably 0.1 μm to 500 μm , without being particularly limited thereto.

[0018] In step c) of the present invention, the solution may be concentrated by any means as long as it allows the solution to be concentrated to reach a concentration at which the crosslinking reaction is further facilitated. This concentration step is also intended to include, e.g., a state where the solvent is completely removed such that the crosslinking reaction proceeds as a solid phase reaction.

[0019] Alternatively, the above steps b) and c) may be carried out in a single process. More specifically, the above steps b) and c) may be carried out in a single process by using techniques such as spray drying method, emulsion solvent evaporation method, and solvent diffusion method. Among them, spray drying is preferred for carrying out the above steps b) and c) in a single process.

[0020] The crosslinked polysaccharide microparticles of the present invention can be prepared when a solution containing a polysaccharide derivative having a crosslinkable functional group(s) is concentrated starting from a dilute state, where crosslinking proceeds slowly, to reach a concentration which facilitates crosslinking, to thereby crosslink the polysaccharide derivative during concentration. Likewise, the present invention is also

characterized in providing drug-carrying microparticles that are obtained when a solution containing a drug and a polysaccharide derivative having a crosslinkable functional group(s) is concentrated starting from a dilute state,

5 where crosslinking proceeds slowly, to reach a concentration which facilitates crosslinking, to thereby cause crosslinking reaction during concentration, in parallel with drying, so that the drug is encapsulated into the crosslinked polysaccharide.

10 [0021] The method provided by the present invention and the crosslinked polysaccharide microparticles (e.g., crosslinked hyaluronic acid microparticles) obtained by the method preferably have characteristics as shown below.

15 1. The method and microparticles ensure complete biodegradability and *in vivo* safety.

2. By grafting a polysaccharide such as HA with a crosslinkable functional group(s), the method and microparticles can keep a very short distance between crosslinking points (e.g., in the case of HA, about 3 nm 20 when grafted at 33 mol% based on glucuronic acid) and are advantageous in achieving long-term sustained release.

3. The method and microparticles achieve a high crosslinking density.

25 4. When a protein is used as a drug, the method and microparticles can prevent protein denaturation.

5. Microparticle formation, drying and crosslinking can be carried out in a single process.

[0022] As used herein, the term "crosslinking" or

"chemical crosslinking" is intended to mean containing intermolecular or intramolecular crosslinkages via covalent bonds. It may also mean having both intermolecular and intramolecular crosslinkages.

5 [0023] The crosslinking reaction used in the present invention is not limited in any way as long as it allows crosslinkage formation without causing drug denaturation even when crosslinkages are formed in the presence of drugs such as proteins or peptides. Examples of such a reaction
10 include disulfide formation between mercapto groups, addition reaction between a mercapto group and an unsaturated bond, and reaction between a hydrazide group and an activated carboxylic acid ester.

[0024] The pH during crosslinking is not limited in
15 any way, but it is preferably a pH at which crosslinkage formation is facilitated without causing protein or peptide denaturation so as to prevent reactions with amino groups contained in drugs such as proteins or peptides. Although such a pH can be selected as appropriate by those skilled
20 in the art, it ranges from, for example, pH 3.0 to pH 9.0, and preferably pH 4.5 to pH 9.0.

[0025] The polysaccharide derivative used in the present invention is not limited in any way as long as it is crosslinkable as described above. Specific examples
25 include hyaluronic acid derivatives (HA derivatives) having a crosslinkable functional group(s) introduced into HA. The crosslinkable functional groups used in the present invention include, without any limitation, a mercapto group,

an unsaturated bond-containing group (e.g., a methacryl group, an acryl group, a vinylsulfone group, an acetylenecarbonyl group), and a hydrazide group (HZ group).

[0026] In a case where the crosslinking reaction is due to disulfide formation between mercapto groups, for example, crosslinkages can be formed using a polysaccharide derivative such as a HA derivative having mercapto groups, either alone or in combination with a crosslinking agent such as a compound having two or more mercapto groups (e.g., 10 dithiothreitol (DTT), butanedithiol, polyethylene glycol dithiol, a peptide having two or more cysteines). With the aim of achieving a higher reaction rate of crosslinking, compounds such as sodium tetrathionate (STT), dipyridyl disulfide and Ellman's reagent (DTNB) may be added. In 15 this case, if unreacted mercapto groups remain in the resulting gel, they have a possibility of leading to protein or peptide denaturation. Thus, to maximize the reaction efficiency, these compounds may preferably be added in a 0.1- to 2-fold molar amount, more preferably in 20 a 0.5- to 1.5-fold molar amount, relative to reactive mercapto groups.

[0027] Although there is no particular limitation on the method for preparing a polysaccharide derivative having mercapto groups, for example, HA may be converted into a 25 tertiary ammonium salt form, dissolved in a polar organic solvent such as DMSO, and then reacted with a mercapto group-containing amine or hydrazide in the presence of a coupling agent. Examples of such a mercapto group-

containing amine include, without any limitation,
2-aminoethane-1-thiol, 3-aminopropane-1-thiol, and
thioglycolic acid hydrazide.

[0028] In the case of introducing mercapto groups into
5 HA, it is also preferable to first introduce an amino group
or a hydrazide group and then introduce a mercapto group
into this amino or hydrazide group. For example,
carboxylic acid in HA may be condensed with adipic acid
dihydrazide (ADH) or a divalent compound containing HZ or
10 amino groups (e.g., ethylenediamine,
ethylenedioxybisethylamine) in the presence of a condensing
agent to synthesize a hydrazide group-modified HA
derivative (HA-HZ) or an amino group-modified HA derivative
(HA-amino group), which may then be reacted with, e.g.,
15 N-succinimidyl 3-[2-pyridyldithio]propionate (SPDP) and
treated with a reducing agent (e.g., DTT) to form mercapto
groups, or alternatively, which may then be reacted with
2-iminothiolane (Trout's Reagent) at the hydrazide or amino
groups.

20 [0029] Examples of a coupling agent include
benzotriazol-1-yloxy-tris(dimethylamino)phosphonium
hexafluorophosphate (BOP), benzotriazol-1-yloxy-
trispyrrolidinophosphonium hexafluorophosphate (PyBOP),
N,N'-carbonyldiimidazole (CDI), N,N'-
25 dicyclohexylcarbodiimide (DCC), 1-ethyl-3-(3-
dimethylaminopropyl)carbodiimide hydrochloride (EDC),
EDC/3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine
(HODhbt), N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline

(EEDQ), 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholium chloride n-hydrate (DMT-MM), and 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU).

5 [0030] The crosslinkable functional groups intended in the present invention may be introduced, for example, by converting carboxyl groups contained in polysaccharide molecules into mercapto group-, unsaturated bond-, amino group- or hydrazyl group-containing ester or substituted
10 amido groups as shown below:

-CO-N(-R₁)-Y₁-Q₁-Y₂-N(-R₂)-Y₃-Q₂-SH;

-CO-N(-R₁)-N(-R₂)-Y₃-Q₂-SH;

-CO-N(-R₁)-Y₁-Q₁-Y₂-N(-R₂)-Y₃-Q₄;

-CO-N(-R₁)-N(-R₂)-Y₃-Q₄; or

15 -CO-N(-R₁)-Y₁-Q₁-Y₂-NH₂;

(wherein R₁ represents a hydrogen atom, a linear or branched C₁₋₁₀ alkyl group, a linear or branched C₁₋₁₀ hydroxyalkyl group, a polyalkylene oxide group, a polypeptide group or a polyester group.

20 Y₁ represents a single bond, -N(-R₃)CO-, -N(-R₃)-, -CO- or -CH₂CO-,

Y₂ represents a single bond, -CON(-R₄)- or -N(-R₄)-,

Q₁ represents a linear or branched C₁₋₁₀ alkylene group, a linear or branched C₁₋₁₀ hydroxyalkylene group, a polyalkylene oxide group, a polypeptide group or a polyester group,
25 R₂, R₃ and R₄ each independently represent a hydrogen atom, a linear or branched C₁₋₁₀ alkyl group, a linear or

R₂, R₃ and R₄ each independently represent a hydrogen atom, a linear or branched C₁₋₁₀ alkyl group, a linear or

branched C₁₋₁₀ hydroxyalkyl group, a polyalkylene oxide group, a polypeptide group or a polyester group,

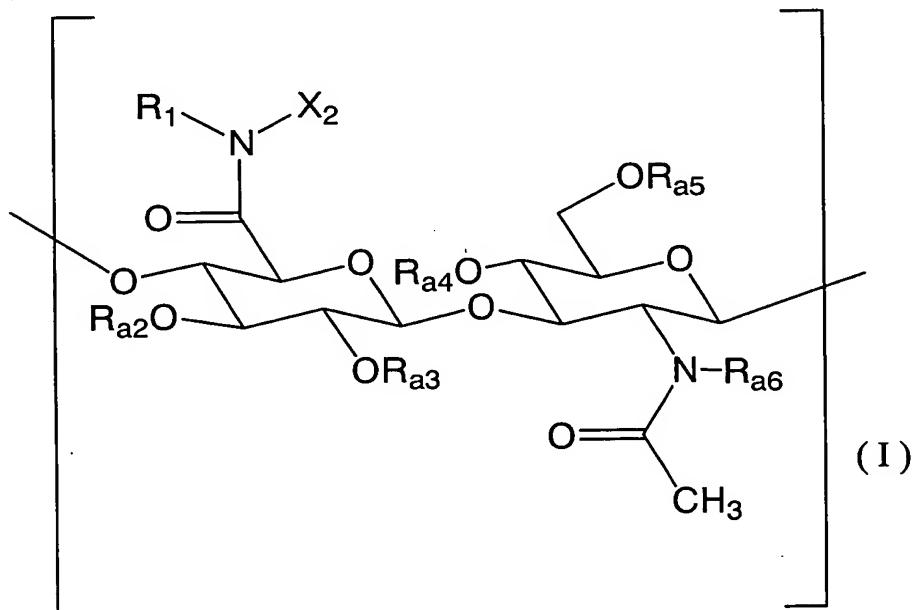
Y₃ represents a single bond, -CO-, -CO₂-, -CH₂-CH(OH)- or -CONH-,

5 Q₂ represents a linear or branched C₁₋₁₀ alkylene group, a linear or branched C₁₋₁₀ hydroxyalkylene group, a polyalkylene oxide group, a polypeptide group or a polyester group, and

10 Q₄ represents a linear or branched C₂₋₁₀ alkenyl group or a linear or branched C₂₋₁₀ alkynyl group).

[0031] Examples of a polysaccharide derivative having mercapto groups preferably include a hyaluronic acid derivative whose molecule contains at least one or more repeated structures represented by Formula (I):

15 [0032] [Formula 1]



[0033] (wherein X₂ represents -Y₁-Q₁-Y₂-N(-R₂)-Y₃-Q₂-SH or -N(-R₂)-Y₃-Q₂-SH,

R₁ represents a hydrogen atom, a linear or branched C₁₋₁₀ alkyl group, a linear or branched C₁₋₁₀ hydroxyalkyl group, a polyalkylene oxide group, a polypeptide group or a polyester group.

5 R_{a2}, R_{a3}, R_{a4}, R_{a5} and R_{a6} each independently represent a hydrogen atom, a linear or branched C₁₋₆ alkyl group, a linear or branched C₁₋₆ alkenyl group, a linear or branched C₁₋₆ alkynyl group, a linear or branched C₁₋₆ alkylcarbonyl group, a linear or branched C₁₋₆ alkenylcarbonyl group, a 10 linear or branched C₁₋₆ alkynylcarbonyl group or -SO₂OH,

Y₁ represents a single bond, -N(-R₃)CO-, -N(-R₃)-, -CO- or -CH₂CO-,

Y₂ represents a single bond, -CON(-R₄)- or -N(-R₄)-,

Q₁ represents a linear or branched C₁₋₁₀ alkylene group, a linear or branched C₁₋₁₀ hydroxyalkylene group, a polyalkylene oxide group, a polypeptide group or a 15 polyester group,

R₂, R₃ and R₄ each independently represent a hydrogen atom, a linear or branched C₁₋₁₀ alkyl group, a linear or 20 branched C₁₋₁₀ hydroxyalkyl group, a polyalkylene oxide group, a polypeptide group or a polyester group,

[0034] Y₃ represents a single bond, -CO-, -CO₂-, -CH₂-CH(OH)- or -CONH-, and

Q₂ represents a linear or branched C₁₋₁₀ alkylene group, a 25 linear or branched C₁₋₁₀ hydroxyalkylene group, a polyalkylene oxide group, a polypeptide group or a polyester group).

[0035] As used in Formula (I), the term "polyalkylene

oxide group" refers to a group represented by -(CH(-R)CH₂O)_n-H (wherein R represents a hydrogen atom or a C₁₋₅ alkyl group) and preferably represents a polyethylene oxide group or a polypropylene oxide group. Likewise, n is

5 preferably an integer of 1 to 20. Although the polypeptide group is not limited in any way, it is preferably composed of 1 to 20 amino acids. Likewise, although the polyester group is not limited in any way, it is preferably a polyglycolic acid group or a polylactic acid group.

10 [0036] Moreover, in Formula (I), R₁ preferably represents a hydrogen atom, and X₂ preferably represents -Y₁-Q₁-Y₂-N(-R₂)-Y₃-Q₂-SH. In Formula (II), Y₁ preferably represents a single bond or -N(-R₃)-, Y₂ preferably represents a single bond, and Q₁ preferably represents a

15 linear or branched C₁₋₄ alkylene group. In Formula (II), R₂ and R₃ each preferably represent a hydrogen atom, Y₃ preferably represents -CO-, and Q₂ preferably represents a linear or branched C₁₋₄ alkylene group.

[0037] In a case where addition reaction between a

20 mercapto group and an unsaturated bond is utilized for crosslinking reaction, a polysaccharide derivative such as a HA derivative having unsaturated bond-containing groups may be mixed with a compound having two or more mercapto groups (e.g., dithiothreitol (DTT), butanedithiol,

25 polyethylene glycol dithiol, a peptide having two or more cysteines, a mercapto group-modified HA derivative), or contrariwise, a polysaccharide derivative having mercapto groups may be mixed with a compound having two or more

unsaturated bond-containing groups (e.g., ethylene glycol dimethacrylate, ethylenebisacrylamide, tris-2-maleimidooethylamine, 1,8-bismaleimidotriethylene glycol, 1,4-bismaleimidyl-2,3-dihydroxybutane, a HA derivative having unsaturated bonds). In this case, it is preferable to add a basic compound such as triethanolamine for the purpose of improving the stability of proteins or peptides during crosslinking reaction and improving the reaction rate. The concentration preferred for this purpose is 10 $\mu\text{L/mL}$ to 20 $\mu\text{L/mL}$. Examples of a compound having two or more mercapto groups also include a linear or branched C₂₋₁₀ alkylenedithiol (whose alkylene moiety may have one or more oxygen atoms inserted therein and/or may be substituted with one or more hydroxyl groups).

15 [0038] There is no particular limitation on the method for preparing a polysaccharide derivative having unsaturated groups, but it is difficult to achieve a high introduction rate, e.g., when glycidyl ether methacrylate or methacrylic anhydride is directly reacted with a 20 hydroxyl group in HA (J. Biomed. Mat. Res. 54, 115-121, 2001). This is because HA will form a higher-order structure in an aqueous solution by the action of hydrogen bonding or hydrophobic interaction to reduce the reactivity of its functional groups including hydroxyl and carboxylic acid groups. A higher crosslinking density is desired to 25 achieve prolonged sustained release of proteins or peptides. To this end, it is desirable to introduce a substituent at the carboxyl group of the glucuronic acid moiety. For

example, HA may be converted into a tertiary ammonium salt form, dissolved in a polar organic solvent such as DMSO, and then reacted with an unsaturated bond-containing amine or hydrazide in the presence of a coupling agent such as
5 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), benzotriazol-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP) or benzotriazol-1-yloxy-trispyrrolidinophosphonium hexafluorophosphate (PyBOP) to prepare a desired derivative. Examples of such an
10 unsaturated bond-containing amine include, without any limitation, allylamine, diallylamine, 4-amino-1-butene, acrylhydrazide, and methacrylhydrazide.

[0039] It is also preferable to introduce an amino group or a hydrazide group and then introduce an
15 unsaturated bond-containing group into this amino or hydrazide group, as described above. For example, carboxylic acid in HA may be condensed with adipic acid dihydrazide (ADH) or a divalent compound containing HZ or amino groups (e.g., ethylenediamine,
20 ethylenedioxybisethylamine) in the presence of a condensing agent (e.g., EDC, BOP, PyBOP) to synthesize a hydrazide group-modified HA derivative (HA-HZ) or an amino group-modified HA derivative (HA-amino group), which may then be reacted with an unsaturated bond-containing carboxylic acid derivative such as an acid anhydride or activated ester of
25 R₁₀-COOH (wherein R₁₀ represents a linear or branched C₂₋₁₀ alkenyl group), preferably with methacrylic anhydride, N-hydroxysuccinimide (NHS)-activated acrylic acid,

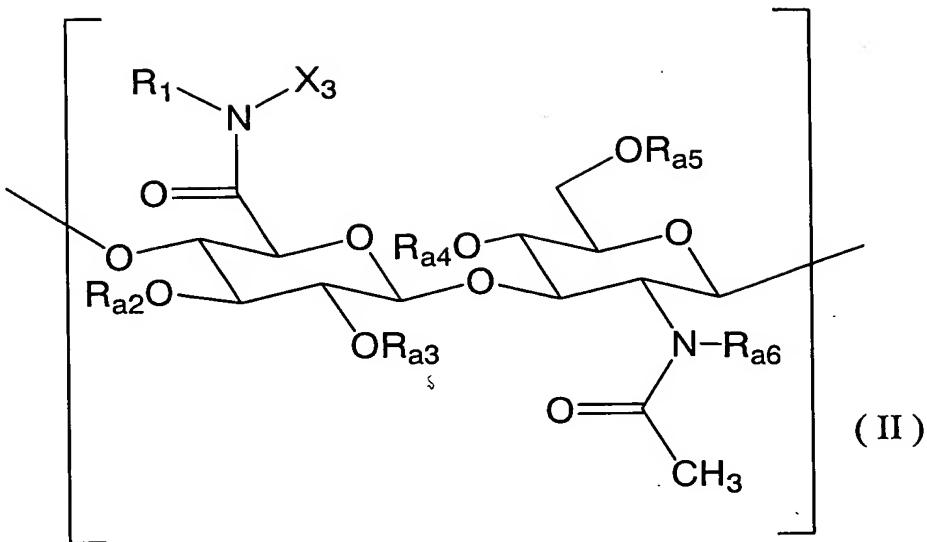
methacrylic acid or the like.

[0040] In a case where polysaccharide (e.g., HA) molecules are crosslinked via mercapto groups after introduction of unsaturated bond-containing groups, the 5 ratio of mercapto groups to unsaturated bond-containing groups is not limited in any way and can be selected as appropriate by those skilled in the art. However, the ratio of mercapto groups to unsaturated bond-containing groups is preferably 3:1 to 1:2, more preferably 2:1 to 1:1, 10 in order to minimize reactions with proteins or peptides, to prevent unsaturated groups from remaining in the gel and to ensure rapid reaction.

[0041] In a case where HA molecules are crosslinked via unsaturated bond-containing groups after introduction 15 of mercapto groups, the ratio of unsaturated bond-containing groups to mercapto groups is not limited in any way and can be selected as appropriate by those skilled in the art. However, the ratio of unsaturated bond-containing groups to mercapto groups is preferably 3:1 to 1:2, more preferably 2:1 to 1:1, in order to minimize reactions with 20 proteins or peptides, to prevent unsaturated groups from remaining in the gel and to ensure rapid reaction.

[0042] Examples of a polysaccharide derivative having unsaturated bond-containing groups preferably include a 25 hyaluronic acid derivative whose molecule contains at least one or more repeated structures represented by Formula (II):

[0043] [Formula 2]



[0044] (wherein X_3 represents $-Y_1-Q_1-Y_2-N(-R_2)-Y_3-Q_4$ or
 $-N(-R_2)-Y_3-Q_4$,

R_1 represents a hydrogen atom, a linear or branched
5 C_{1-10} alkyl group, a linear or branched C_{1-10} hydroxyalkyl
group, a polyalkylene oxide group, a polypeptide group or a
polyester group,

R_{a2} , R_{a3} , R_{a4} , R_{a5} and R_{a6} each independently represent
a hydrogen atom, a linear or branched C_{1-6} alkyl group, a
10 linear or branched C_{1-6} alkenyl group, a linear or branched
 C_{1-6} alkynyl group, a linear or branched C_{1-6} alkylcarbonyl
group, a linear or branched C_{1-6} alkenylcarbonyl group, a
linear or branched C_{1-6} alkynylcarbonyl group or $-SO_2OH$.

Y_1 represents a single bond, $-N(-R_3)CO-$, $-N(-R_3)-$,
15 $CO-$ or $-CH_2CO-$,

Y_2 represents a single bond, $-CON(-R_4)-$ or $-N(-R_4)-$,

Y_3 represents a single bond, $-CO-$ or $-CH_2CO-$,

Q_1 represents a linear or branched C_{1-10} alkylene
group, a linear or branched C_{1-10} hydroxyalkylene group, a

polyalkylene oxide group, a polypeptide group or a polyester group.

R₂, R₃ and R₄ each independently represent a hydrogen atom, a linear or branched C₁₋₁₀ alkyl group, a linear or branched C₁₋₁₀ hydroxyalkyl group, a polyalkylene oxide group, a polypeptide group or a polyester group, and

Q₄ represents a linear or branched C₂₋₁₀ alkenyl group or a linear or branched C₂₋₁₀ alkynyl group).

[0045] As used in Formula (II), the term "polyalkylene oxide group" refers to a group represented by -(CH(-R)CH₂O)_n-H (wherein R represents a hydrogen atom or a C₁₋₅ alkyl group) and preferably represents a polyethylene oxide group or a propylene oxide group. Likewise, n is preferably an integer of 1 to 20. Although the polypeptide group is not limited in any way, it is preferably composed of 1 to 20 amino acids. Likewise, although the polyester group is not limited in any way, it is preferably a polyglycolic acid group or a polylactic acid group.

[0046] Moreover, in Formula (II), R₁ preferably represents a hydrogen atom, and X₃ preferably represents -Y₁-Q₁-Y₂-N(-R₂)-Y₃-Q₄. In Formula (II), Y₁ preferably represents a single bond, -N(-R₃)CO- or -N(-R₃)-, more preferably -N(-R₃)CO-. Likewise, Y₂ preferably represents a single bond or -CON(-R₃)-, more preferably -CON(-R₃)-, and Y₃ preferably represents a single bond, -CO- or -N(-R₃)-, more preferably -CO-. In Formula (II), Q₁ preferably represents a linear or branched C₁₋₄ alkylene group, R₂ and R₃ each preferably represent a hydrogen atom, and Q₄

preferably represents a linear or branched C₂₋₁₀ alkenyl group.

[0047] Likewise, examples of a polysaccharide derivative having mercapto groups include a hyaluronic acid derivative whose molecule contains at least one or more repeated structures represented by Formula (I) as mentioned above.

[0048] Reaction between a polysaccharide derivative such as a HA derivative having hydrazide groups and activated carboxylic acid may also be used for crosslinking reaction. Introduction of hydrazide groups into a polysaccharide may be accomplished in any manner known to those skilled in the art. For example, a carboxyl group in hyaluronic acid may be condensed with a divalent hydrazide-containing compound (i.e., a dihydrazide compound) in the presence of a condensing agent to synthesize a desired derivative. Examples of a dihydrazide compound include succinic acid dihydrazide, glutaric acid dihydrazide, adipic acid dihydrazide, and pimelic acid dihydrazide.

Likewise, examples of a condensing agent include 1,3-dicyclohexylcarbodiimide, 1,3-diisopropylcarbodiimide, and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. For example, carboxylic acid in hyaluronic acid may be condensed with adipic acid dihydrazide (ADH) in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) to synthesize a hydrazide group-modified hyaluronic acid (HA-HZ). The crosslinking agent is not limited in any way as long as it has a functional group capable of reacting with a HZ group.

and examples include molecules having, in the same molecule, two or more functional groups such as an NHS-activated ester group, a pentafluorophenoxy carbonyl group, a p-nitrophenoxy carbonyl group, an imidazolyl carbonyl group, an 5 isothiocyanato group, a sulfonyl chloride group, a sulfonyl fluoride group, a formyl group, a vinylsulfonyl group, an acid anhydride and a 4-nitrophenylformate group.

Illustrative examples of such a crosslinking agent include bis[sulfosuccinimidyl]suberate, disuccinimidyl glutarate, 10 disuccinimidyl tartrate, and ethylene glycol bis[succinimidylsuccinate].

[0049] Taking into account the selective reactivity with a HZ group relative to an amino group, protein denaturation and the like, the pH during crosslinking is 15 preferably pH 3.0 to pH 6.0, and more preferably pH 4.0 to pH 6.0. To maintain the pH within this range during crosslinking, the buffer to be used is preferably a less volatile one, as exemplified by citrate buffer. The crosslinking agent is used such that the amount of its functional groups capable of reacting with a hydrazide 20 group is preferably 40 mol% or less, more preferably 20 mol% or less, particularly preferably 10 mol% or less, relative to hydrazide groups in a gel preparation solution.

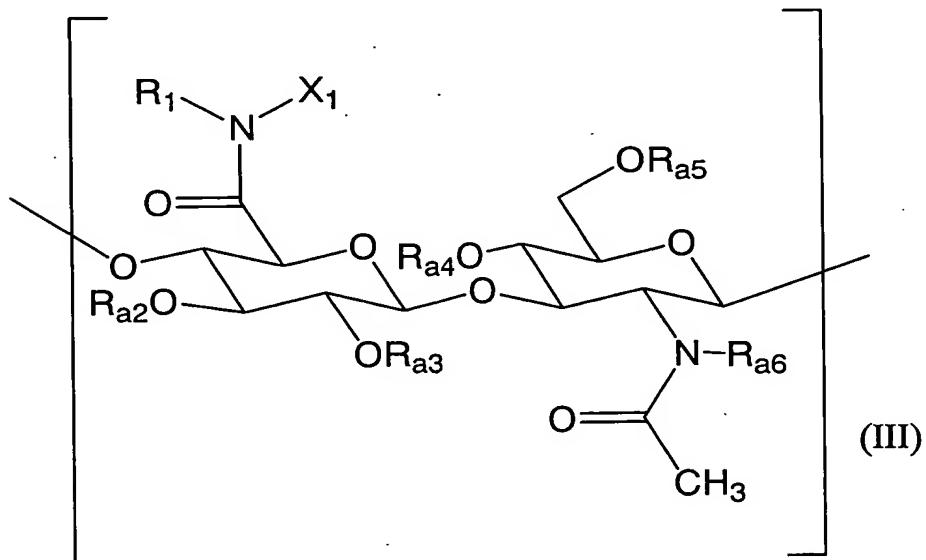
[0050] Although there is no particular limitation on 25 the rate of crosslinkable functional groups introduced into HA, it is preferably 5 mol% or more, particularly preferably 10 mol% or more, based on glucuronic acid in HA in order to obtain a non-flowable gel *in vivo*. Moreover,

since the sustained release performance of a drug greatly depends on the crosslinking density of a crosslinked HA derivative, the sustained release period of the drug can be controlled by regulating this introduction rate.

- 5 [0051] Examples of a polysaccharide derivative having hydrazide groups include a hyaluronic acid derivative whose molecule contains at least one or more repeated structures represented by Formula (III):

[0052] [Formula 3]

10



- [0053] (wherein R_1 represents a hydrogen atom, a linear or branched C_{1-10} alkyl group, a linear or branched C_{1-10} hydroxyalkyl group, a polyalkylene oxide group, a polypeptide group or a polyester group,

15 X_1 represents $-Y_1-Q_1-Y_2-\text{NHNH}_2$,
 R_{a2} , R_{a3} , R_{a4} , R_{a5} and R_{a6} each independently represent a hydrogen atom, a linear or branched C_{1-6} alkyl group, a linear or branched C_{1-6} alkenyl group, a linear or branched

C_{1-6} alkynyl group, a linear or branched C_{1-6} alkylcarbonyl group, a linear or branched C_{1-6} alkenylcarbonyl group, a linear or branched C_{1-6} alkynylcarbonyl group or $-SO_2OH$,

Y_1 represents a single bond, $-N(-R_3)CO-$, $-N(-R_3)-$, -

5 $CO-$ or $-CH_2CO-$,

Q_1 represents a single bond, a linear or branched C_{1-10} alkylene group, a linear or branched C_{1-10} hydroxyalkylene group, a polyalkylene oxide group, a polypeptide group or a polyester group,

10 Y_2 represents a single bond, $-N(-R_4)CO-$, $-CO-$ or -
 CH_2CO- , and

R_3 and R_4 each independently represent a hydrogen atom, a linear or branched C_{1-10} alkyl group, a linear or branched C_{1-10} hydroxyalkyl group, a polyalkylene oxide group, a polypeptide group or a polyester group).

[0054] Moreover, in Formula (III), R_1 preferably represents a hydrogen atom, R_{a2} , R_{a3} , R_{a4} , R_{a5} and R_{a6} each preferably represent a hydrogen atom, Y_1 preferably represents a single bond or $-CO-$, Q_1 preferably represents a linear or branched C_{1-10} alkylene group, Y_2 preferably represents a single bond or $-CO-$, R_3 preferably represents a hydrogen atom, and R_4 preferably represents a hydrogen atom.

[0055] As used in Formula (III), the term
25 "polyalkylene oxide group" refers to a group represented by $-(CH(-R)CH_2O)_n-OH$ (wherein R represents a hydrogen atom or a linear or branched C_{1-5} alkyl group) and preferably represents a polyethylene oxide group or a polypropylene

oxide group. Likewise, n is preferably an integer of 1 to 20. Although the polypeptide group is not limited in any way, it is preferably composed of 1 to 20 amino acids. Likewise, although the polyester group is not limited in any way, it is preferably a polyglycolic acid group or a 5 polylactic acid group.

[0056] The method of the present invention for preparing crosslinked hyaluronic acid microparticles may be any method in which crosslinking proceeds in parallel with 10 drying of microparticles through solvent distillation. For example, by using a spray dryer for spraying and drying a fluid, a solution containing a drug and a hyaluronic acid derivative having a crosslinkable functional group(s) may be sprayed and dried so as to crosslink the hyaluronic acid 15 derivative during concentration and drying, thereby obtaining drug-carrying microparticles in which the drug is encapsulated in the crosslinked hyaluronic acid. In the case of using the spray-dry technique, the drying temperature is preferably set at 100°C or below in order to 20 prevent drug denaturation.

[0057] Alternatively, a crosslinkable HA derivative (in a tetrabutylammonium salt form) and a drug may be dissolved in a polar organic solvent such as DMSO, followed by addition of a supercritical fluid such as carbon dioxide. 25 DMSO may then be extracted to cause crosslinking reaction during concentration of hyaluronic acid, thereby obtaining microparticles. When using these techniques for microparticle formation, the addition of a surfactant such

as Tween-20 or Tween-80 (around 1-2%) enables improvement in the recovery rate of the resulting microparticles. These techniques also require the use of a preparation solution which does not cause crosslinking reaction before 5 concentration. The introduction rate of crosslinkable functional groups is preferably 5 mol% to 70 mol%, the molecular weight of hyaluronic acid is preferably 10,000 to 2,000,000 daltons, and the concentration of hyaluronic acid is preferably 0.1% to 5%, although they will vary depending 10 on the time required for a process starting from mixing with a crosslinking agent until concentration, the introduction rate of crosslinkable functional groups, as well as on the molecular weight and concentration of hyaluronic acid.

15 [0058] In another method, an aqueous solution containing a drug and a hyaluronic acid derivative having a crosslinkable functional group(s) may also be emulsified in a dehydrating fluid (e.g., polyethylene glycol with a molecular weight of 400 daltons) to effect crosslinking of 20 hyaluronic acid during dehydration/concentration, thereby obtaining drug-carrying microparticles in which the drug is encapsulated in the crosslinked hyaluronic acid. When using this method, a cationic or nonionic drug is preferred to improve the encapsulation efficiency.

25 [0059] Moreover, it is more preferable to perform thermal treatment after microparticle formation, so that the water content is further reduced and the crosslinking reaction is completely terminated. In this case, the

crosslinking density is also improved and a prolonged sustained release can also be expected. The temperature required for thermal treatment is not limited in any way, but it may be performed, for example, at 30°C to 110°C, 5 preferably at 30°C to 60°C.

[0060] Although the diameter of the dried microparticles may be optimized for their intended use, the diameter preferred for injectable purposes is usually 0.01 µm to 150 µm. For transnasal or transpulmonary 10 administration, a diameter of 0.01 µm to 5 µm is preferred in terms of inhalation efficiency, while a diameter of around 0.01 µm to 0.2 µm is preferred for intravenous injection in terms of blood pharmacokinetics.

[0061] HA used in the present invention may be of any 15 origin, including HA extracted from animal tissues, HA obtained by fermentation techniques, and HA chemically synthesized. Moreover, further treatment (e.g., hydrolysis) may be performed on HA. Modified HA prepared in various manners and its salts with alkali metals (e.g., 20 sodium, potassium, lithium) also fall within the scope of HA according to the present invention. Although HA is often modified at its carboxyl or hydroxyl groups, the modified HA according to the present invention may be modified at any moiety. Such modified HA is not limited in 25 any way and may receive any modification. Examples include sulfated HA (WO95/25751), N-sulfated HA (WO98/45335), esterified HA (EP0216453, WO98/08876, EP0341745), periodate-oxidized HA, and amide-modified HA.

- [0062] There is no particular limitation on the molecular weight of HA used as a starting material in the present invention, and it is possible to use HA of any molecular weight. In general, HA of 5,000 to 3,500,000 daltons, preferably 10,000 to 1,000,000 daltons, can be used. The molecular weight and concentration of HA will affect the particle diameter of the resulting microparticles and hence may be selected as appropriate for the intended particle diameter.
- [0063] Examples of a pharmacologically active protein or peptide include, without any limitation, erythropoietin (EPO), granulocyte colony-stimulating factor (G-CSF), interferons- α , β and γ (INF- α , β , γ), thrombopoietin (TPO), ciliary neurotrophic factor (CNTF), tumor necrosis factor binding protein (TNFbp), interleukin-10 (IL-10), FMS-like tyrosine kinase (Flt-3), growth hormone (GH), insulin, insulin-like growth factor-1 (IGF-1), platelet-derived growth factor (PDGF), interleukin-1 receptor antagonist (IL-1ra), brain-derived neurotrophic factor (BDNF), keratinocyte growth factor (KGF), stem cell factor (SCF), megakaryocyte growth and development factor (MGDF), osteoprotegerin (OPG), leptin, parathyroid hormone (PTH), basic fibroblast growth factor (b-FGF), bone morphogenetic protein (BMP), atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), C-type natriuretic peptide (CNP), glucagon-like peptide-1 (GLP-1), antibody, and diabody. Moreover, the sustained-release drug carrier of the present invention can also be used for drugs of low-molecular-

weight compounds. Examples of such low-molecular drugs include carcinostatic agents (e.g., alkylating agents, antimetabolites, alkaloids), immunosuppressive agents, anti-inflammatory agents (e.g., steroid drugs, non-steroidal anti-inflammatory agents), antirheumatic agents, and antibacterial agents (e.g., β -lactam antibiotics, aminoglycoside antibiotics, macrolide antibiotics, tetracycline antibiotics, novel quinolone antibiotics, sulfa drugs).

10 [0064] The sustained-release carrier of the present invention may be administered as a pharmaceutical composition in any dosage form suitable for the intended route of administration, in combination with one or more pharmaceutically acceptable diluents, wetting agents, emulsifiers, dispersants, auxiliaries, antiseptics, buffers, binders, stabilizers and the like. The route of administration may be either parenteral or oral.

BRIEF DESCRIPTION OF DRAWINGS

[0065] [Figure 1] One example of a microscopic photograph showing crosslinked HA-SH microhydrogel microparticles.

[Figure 2] One example of a microscopic photograph showing crosslinked HA-SH microhydrogel microparticles swollen in PBS.

[Figure 3] One example of the thermogravimetric results analyzed for an EPO-encapsulated crosslinked HA-SH microhydrogel.

[Figure 4] A graph showing one example of the RP-HPLC results, which shows the amount of EPO recovered from the

crosslinked HA-SH microhydrogel microparticles obtained in Examples 1 to 4. Starting from the bottom, the curves correspond to the microparticles obtained in Examples 1, 2, 3 and 4, respectively.

- 5 [Figure 5] A graph showing the release profile of EPO from the HA gels obtained in Example 3 and Comparative Example 1.
- [Figure 6] One example of the $^1\text{H-NMR}$ results measured for the hyaluronic acid derivative (HA-HZ) obtained in Example 9-1.
- 10 [Figure 7] One example of the $^1\text{H-NMR}$ results measured for the hyaluronic acid derivative (HA-HZ-SH) obtained in Example 9-2.
- [Figure 8] One example of the $^1\text{H-NMR}$ results measured for the hyaluronic acid derivative (HA-HZ-MA) obtained in
- 15 Example 10.
- [Figure 9] One example of the $^1\text{H-NMR}$ results measured for the hyaluronic acid derivative (HA-AM) obtained in Example 11-1.
- [Figure 10] One example of the $^1\text{H-NMR}$ results measured for the hyaluronic acid derivative (HA-AM-SH) obtained in
- 20 Example 11-2.
- [Figure 11] One example of the $^1\text{H-NMR}$ results measured for the hyaluronic acid derivative (HA-AM-MA) obtained in Example 11-1.
- 25 [Figure 12] A graph showing curing-induced changes in the water content of the particles obtained in Example 12.
- [Figure 13] A graph showing a curing-induced inhibitory effect on swelling of the particles obtained in Example 12.

EXAMPLES

[0066] Preparation of EPO-encapsulated crosslinked hyaluronic acid microparticles

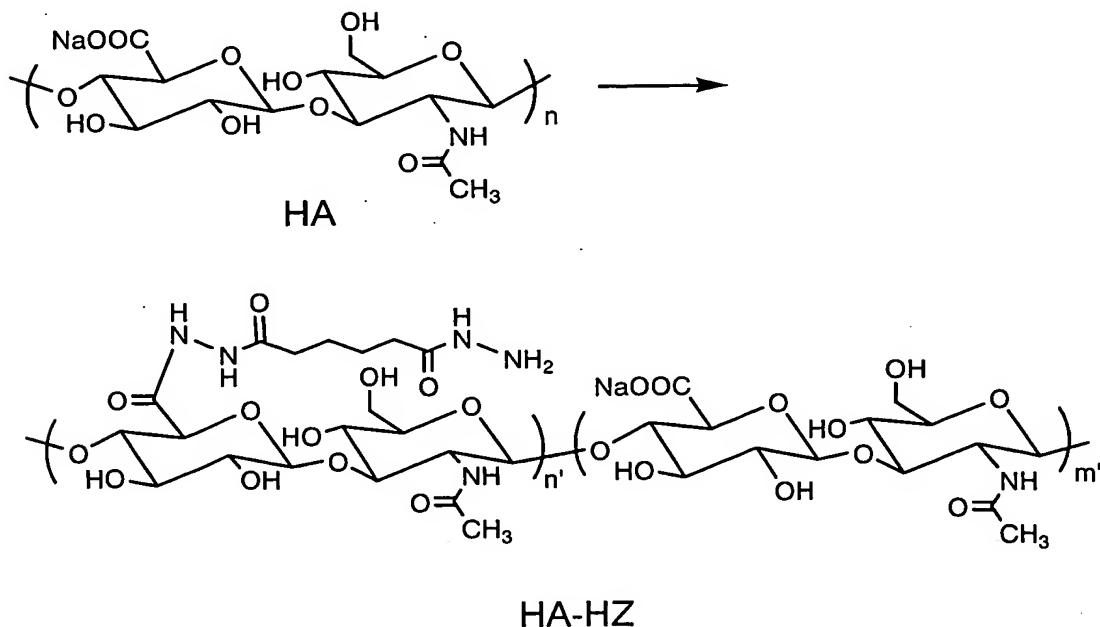
The present invention will be further described in
5 more detail in the following preferred examples, which are
not intended to limit the scope of the invention.

[0067] NMR measurement was carried out using a nuclear magnetic resonance system JNM-ECA500 (JEOL. Ltd., Japan) and heavy water (D_2O) as a solvent. The introduction rate 10 of each substituent was determined from the integral ratio between a peak unique to the introduced substituent and a peak derived from hyaluronic acid.

[Example 1]

[Example 1-1] Synthesis of hydrazide (HZ) group-modified 15 hyaluronic acid derivative (HA-HZ)

[0068] [Formula 4]



[0069] Hyaluronic acid (HA) having a molecular weight

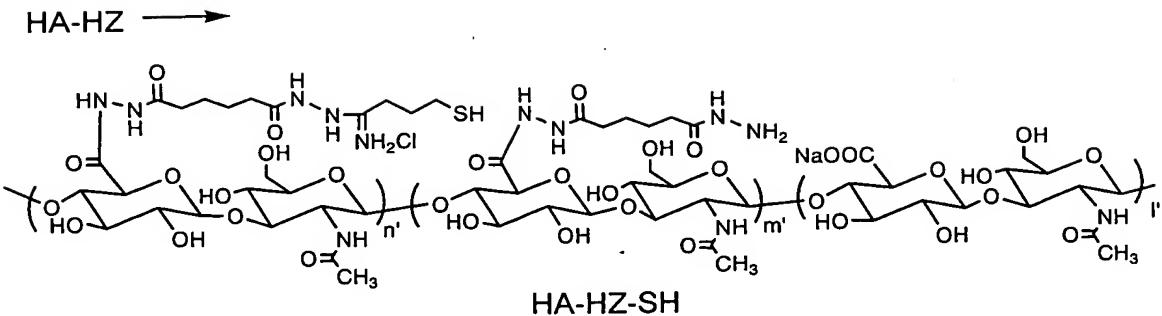
of 1.9×10^5 daltons (200 mg, Denki Kagaku Kogyo Kabushiki Kaisha, Japan) was dissolved in distilled water at a concentration of 0.5% and adjusted with 5N hydrochloric acid to pH 4.7 to 4.8. 1-Ethyl-3-(3-

5 dimethylaminopropyl)carbodiimide (EDC) and adipic acid dihydrazide (ADH) were added at a molar ratio of HA:EDC:ADH = 1:0.3:40 (Batch 1-1), 1:1:40 (Batch 1-2) or 1:5:40 (Batch 1-3), and reacted while stirring at room temperature for 2 hours and adjusting the mixture with 5N hydrochloric acid 10 to maintain a pH of 4.7 to 4.8. The reaction mixture was dialyzed against a 100 mM sodium chloride solution and a 25% ethanol solution (SpectraPor 7, molecular weight cutoff (MWCO): 12k-14k daltons) and lyophilized to give the titled HA-HZ.

15 [0070] The introduction rate of HZ groups in the resulting HA-HZ was determined for each batch by proton NMR, indicating that carboxylic acid in HA was modified with HZ at a rate of 26% (Batch 1-1), 46% (Batch 1-2) or 69% (Batch 1-3) (calculated by comparing N-acetyl groups in HA and 20 HA-HZ (1.9 ppm, 3H) and methylene groups in the adipic acid-derived moiety of HA-HZ (1.6 ppm, 2.3 ppm, 2H each)).

[Example 1-2] Synthesis of mercapto (SH) group-modified hyaluronic acid derivative (HA-SH)

[0071] [Formula 5]



[0072] HA-HZ obtained in Batches 1 to 3 of Example 1-1 (100 mg each) was dissolved in 5 mL of 100 mM phosphate buffer, pH 8 (HA-HZ: 2% w/v), followed by addition of 5 iminothiolane (ITL) (at a molar ratio of HZ/ITL = 1/2). The mixture was reacted while stirring at room temperature for 2 to 4 hours and then precipitated in ethanol, washed three times and dried. The introduction rate of SH groups in the resulting HA-SH was determined for each batch by 10 proton NMR and the results obtained are shown in Table 1 (calculated by comparing N-acetyl groups in HA and HA-SH (1.9 ppm, 3H) and methylene groups in the ITL-derived moiety of HA-SH (2.1 ppm and 2.7 ppm, 2H each)).

[0073] [Table 1]

15 Table 1

	Control	Batch 1	Batch 2	Batch 3
HZ introduction	0%	26%	46%	69%
SH introduction	0%	20%	35%	56%

[0074] [Example 1-3] Preparation of EPO-encapsulated crosslinked hyaluronic acid microparticles

HA-SH (introduction rate of SH groups: 20 mol%)
20 obtained in Batch 1 of Example 1-2 (200 mg) and

- erythropoietin (EPO; 2 mg) were dissolved in 20 mL of 10 mM phosphate buffer (PB; pH 8) (stirring at room temperature for 1 hour), followed by addition of Tween-20 (4 mg) and sodium tetrathionate (STT; 22.3 mg, 1-fold molar amount relative to SH groups). This solution was spray-dried under the following conditions to obtain microparticles.
- Spray dryer: Buchi Mini spray dryer B-191
- Solution feed rate: 1.5 mL/min (Tygon tube, Pump speed = 15%)
- 10 Feed solution concentration: 10 mg/mL
- Atomizing air flow rate: 650 L/hr
- Drying air flow rate: 40 kL/hr (Aspiration speed = 65%)
- Inlet temperature: 85°C to 95°C
- Outlet temperature: 50°C to 60°C
- 15 [Example 2]

The same procedure as shown in Example 1-3 was repeated to prepare EPO-encapsulated crosslinked hyaluronic acid microparticles, except that HA-SH (introduction rate of SH groups: 35 mol%) obtained in Batch 2 (200 mg) and sodium tetrathionate (STT; 39.0 mg, 1-fold molar amount relative to SH groups) were used in the experimental operation of Example 1-3.

[Example 3]

The same procedure as shown in Example 1-3 was repeated to prepare EPO-encapsulated crosslinked hyaluronic acid microparticles, except that HA-SH (introduction rate of SH groups: 56 mol%) obtained in Batch 3 (200 mg) and sodium tetrathionate (STT; 62.4 mg, 1-fold molar amount

relative to SH groups) were used in the experimental operation of Example 1-3.

[Example 4]

The same procedure as shown in Example 1-3 was repeated to prepare EPO-encapsulated crosslinked hyaluronic acid microparticles, except that HA-SH (introduction rate of SH groups: 56 mol%) obtained in Batch 3 (200 mg) and sodium tetrathionate (STT; 38.9 mg, 0.7-fold molar amount relative to SH groups) were used in the experimental operation of Example 1-3.

[Example 5]

The same procedure as shown in Example 1-3 was repeated to prepare EPO-encapsulated crosslinked hyaluronic acid microparticles, except that HA-SH (introduction rate of SH groups: 56 mol%) obtained in Batch 3 (200 mg) and sodium tetrathionate (STT; 27.8 mg, 0.5-fold molar amount relative to SH groups) were used in the experimental operation of Example 1-3.

[Example 6]

The same procedure as shown in Example 3 was repeated to prepare EPO-encapsulated crosslinked hyaluronic acid microparticles, except that Tween-20 (4 mg) was replaced by Tween-80 (4 mg) in the experimental operation of Example 3.

[Example 7]

The same procedure as shown in Example 3 was repeated to prepare EPO-encapsulated crosslinked hyaluronic acid microparticles, except that Tween-20 was not added in the experimental operation of Example 3.

[Example 8]

The same procedure as shown in Example 3 was repeated to prepare EPO-encapsulated crosslinked hyaluronic acid microparticles, except that STT was not added in the 5 experimental operation of Example 3.

[Comparative Example 1]

HA-SH (introduction rate of SH groups: 56 mol%) prepared in Batch 3 of Example 1-2 (33 mg) was dissolved in 690 μ L of 10 mM phosphate buffer (pH 8.0), supplemented 10 with 30 μ L of an aqueous EPO solution (10 mg/mL) and stirred for 10 minutes, followed by addition of a solution prepared by dissolving sodium tetrathionate (STT; 9.3 mg, 1-fold molar amount relative to SH groups) in 30 μ L of 10 mM phosphate buffer, pH 8.0. The resulting solution was 15 filled in 250 μ L volumes into 1 mL syringes and reacted at 37°C for 5 hours to obtain cylindrical HA gels.

[Comparative Example 2]

The same procedure as shown in Example 8 was repeated to prepare EPO-encapsulated HA microparticles, except that 20 HA-SH was replaced by HA in Example 8.

[0075] It should be noted that the microparticles obtained in Examples 1 to 8 and Comparative Example 2 were each collected at a rate of 50% to 65%.

[Test Example 1] Measurement of particle diameter and 25 particle water content

Figure 1 shows a microscopic photograph of the microparticles prepared in Example 3 ($\times 3000$ magnification). Figure 2 shows a microscopic photograph of these

microparticles when dispersed in PBS ($\times 3000$ magnification). The particle diameter of these microparticles was about $1.2 \mu\text{m}$ (dried) and about $1.8 \mu\text{m}$ (swollen with water).

[0076] Thermogravimetry analysis (TGA) was carried out 5 to determine the water content of the microparticles prepared in Example 3 (Figure 3). The water content was about 15%.

[Test Example 2] Measurement of EPO recovery rate in EPO-encapsulated crosslinked hyaluronic acid microparticles

10 The microparticles prepared in Examples 1 to 8 and Comparative Example 2 (5 mg each) were each dispersed in 0.5 mL PBS, supplemented with 0.25 units of Hyaluronidase SD (Hase; Seikagaku Corporation, Japan) and enzymatically treated at 25°C for 3 hours to completely digest the 15 microparticles. Likewise, the gel prepared in Comparative Example 1 (0.25 mL) was supplemented with 0.75 mL PBS (pH 7.4) containing 0.5 units of Hyaluronidase SD (Seikagaku Corporation, Japan) and enzymatically treated at 25°C for 1 day to completely digest the gel. The resulting solutions 20 after enzymatic treatment (0.15 mL each) were used as sample solutions. These sample solutions were measured by reversed-phase chromatography (RP-HPLC) using a 0.1 mg/mL aqueous EPO solution as a standard solution to calculate the EPO concentration in each sample solution from the peak 25 area ratio between standard solution and sample solution. The recovery rate was calculated as the amount of EPO determined by RP-HPLC relative to the amount of EPO added (0.1 mg/gel).

[0077] High performance liquid chromatography analysis on a reversed-phase column (RP-HPLC) was carried out using a Waters 600S controller, a 717 plus autosampler and a 486 infrared absorption spectrometer (Waters) under the
5 following measurement conditions.

Column: C4 (particle diameter: 5 µm, size: 4.6 × 250 mm)

Mobile phases:

A: water/acetonitrile/trifluoroacetic acid = 400/100/1

B: water/acetonitrile/trifluoroacetic acid = 100/400/1

10 Flow rate: 1 mL/minute, eluted with a gradient of the mobile phases A/B = 65/35 to 0/100

Column temperature: around room temperature

Sample temperature: 4°C

Detection wavelength: UV 280 nm

15 Analysis software: Millenium 32 ver. 3.21

When measured as described above, the recovery rate of EPO relative to the initial amount was as follows.

Examples 1 to 4: 90% to 95%

Examples 5 and 6: 80% to 85%

20 Examples 7 and 8: 75% to 80%

Comparative Examples 1 and 2: 90% to 95%

These results confirmed that the recovery rate was improved by addition of STT and/or a surfactant.

[Test Example 3] Sustained release of EPO from the prepared
25 EPO-encapsulated HA hydrogels

The microhydrogel prepared in Example 3 (20 mg) and the bulk gel prepared in Comparative Example 1 (250 µL) were each incubated in 2 mL PBS at 37°C and sampled over

time in 200 μ L aliquots. The amount of EPO released into the buffer was determined by RP-HPLC.

[0078] Figure 1 shows the release profile of EPO from each gel, as determined by assuming that EPO collected from 5 the gel digested with hyaluronidase immediately after gel preparation is set to 100%. After 9 days, hyaluronidase (Hase) was added.

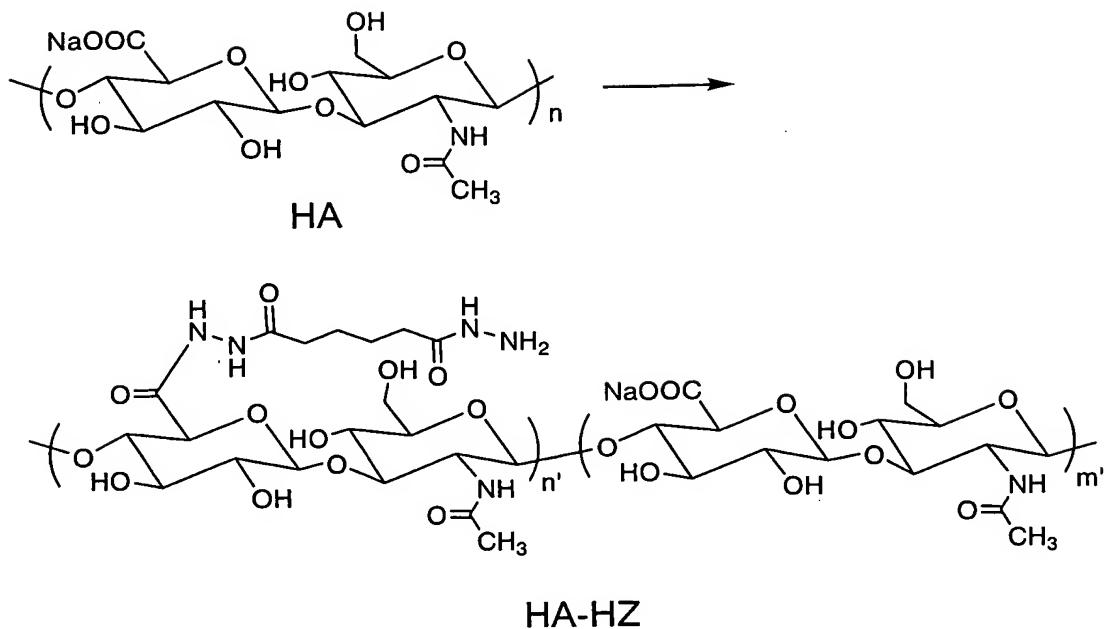
[0079] The results indicate that EPO in the gels remains undenatured and is rapidly released from the gel 10 obtained in Comparative Example 1 because of its low crosslinking density. The results also indicate that the microgel of Example 3 allows sustained release of about 30% EPO over about 5 days because of its high crosslinking density, while 40% of EPO is not released by diffusion but 15 can be released upon enzymatic digestion.

[0080] By using drug-carrying microparticles, in which a drug is encapsulated in crosslinked hyaluronic acid, as illustrated in the above examples, it is possible to prepare injectable sustained-release formulations which 20 allow long-term release of drugs such as proteins or peptides crosslinked *in situ*, dried and encapsulated in gel microparticles while retaining their biological activity.

[Example 9]

[Example 9-1] Synthesis of hydrazide (HZ) group-modified 25 hyaluronic acid derivative HA-HZ (mixed solvent technique)

[0081] [Formula 6]

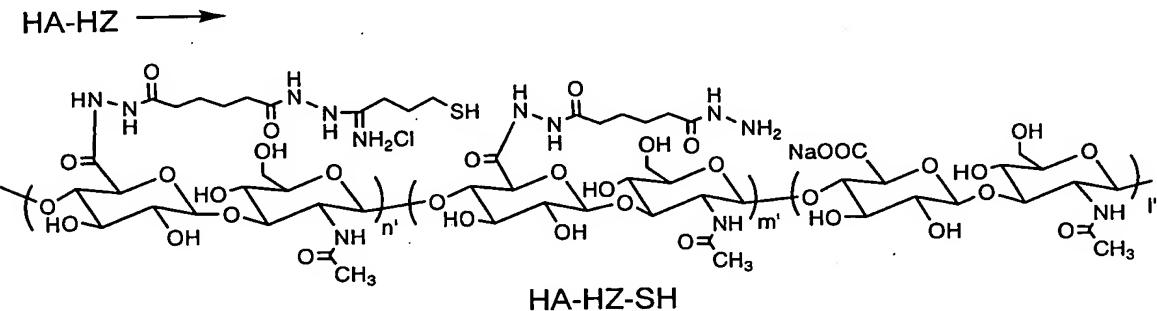


[0082] HA having a molecular weight of 2×10^5 daltons (76.0 mg, Denki Kagaku Kogyo Kabushiki Kaisha, Japan) was dissolved at a concentration of 0.1% in distilled water/EtOH = 50/50 and adjusted with 5N hydrochloric acid to pH 4.7 to 4.8. 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and adipic acid dihydrazide (ADH) were added at a molar ratio of HA unit (1 unit = repeated unit of N-acetylglucosamine-glucuronic acid):EDC:ADH = 1:4:40, and reacted at room temperature for 2 hours while adjusting the mixture with 5N hydrochloric acid to maintain a pH of 4.7 to 4.8. The reaction mixture was dialyzed sequentially against large excess volumes of a 100 mM sodium chloride solution, a 25% ethanol solution and distilled water (SpectraPor 7, molecular weight cutoff (MWCO): 12k-14k daltons) and lyophilized to give 57.0 mg of the titled hydrazide (HZ) group-modified hyaluronic acid (HA-HZ). The

introduction rate of HZ groups in the resulting HA-HZ was determined as the introduction rate of ADH by proton NMR (calculated by comparing N-acetyl groups (1.85 ppm) for HA and ADH-derived 4 methylene groups (1.5, 2.1 and 2.25 ppm) for HZ). The introduction rate of HZ was 47%.

[Example 9-2] Synthesis of mercapto (SH) group-modified hyaluronic acid derivative HA-HZ-SH

[0083] [Formula 7]



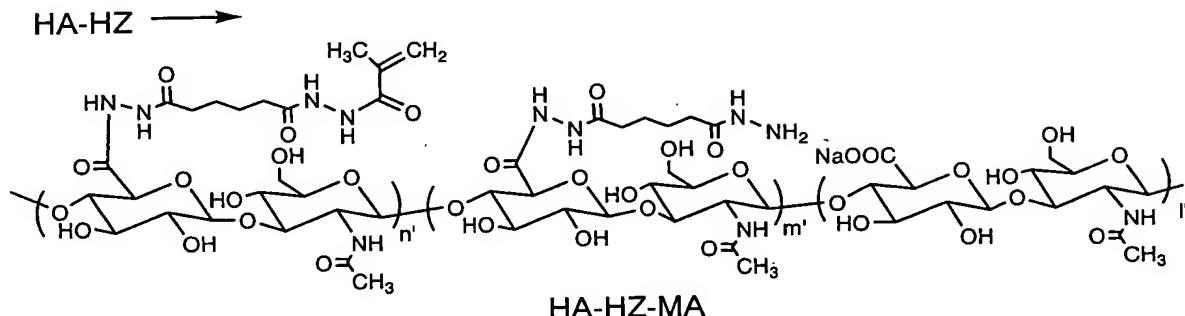
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[0084] HA-HZ synthesized in the same manner as used in Example 9-1 (100 mg) was dissolved in 5 mL of 100 mM phosphate buffer, pH 8 (HA-HZ: 2% w/v), followed by addition of iminothiolane (ITL) (at a molar ratio of HZ/ITL = 1/2). The mixture was reacted while stirring at room temperature for 2 hours and then precipitated in ethanol, washed three times and dried. The introduction rate of SH groups in the resulting HA-HZ-SH was determined by proton NMR, indicating that the introduction rate of SH was 37.5 mol% (calculated by comparing N-acetyl groups in HA and HA-HZ-SH (1.9 ppm, 3H) and methylene groups in the ITL-derived moiety of HA-HZ-SH (2.1 ppm and 2.7 ppm, 2H each)).

[Example 10] Synthesis of methacryloyl (MA) group-modified

hyaluronic acid derivative HA-HZ-MA

[0085] [Formula 8]



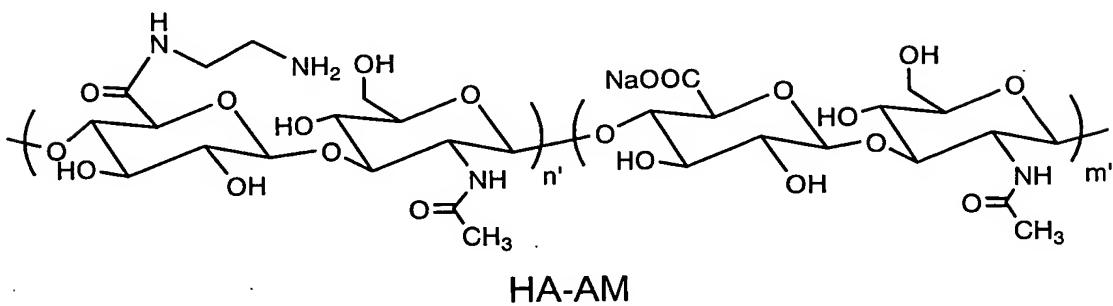
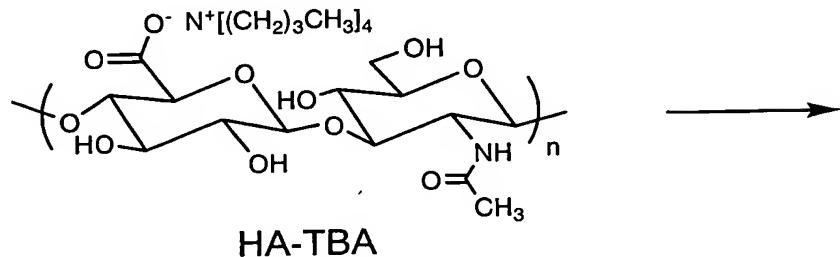
[0086] The same procedure as used in Batch 3 of
5 Example 1-1 was repeated to synthesize HA-HZ (63% of
carboxylic acid in HA was modified with HZ), except that
the molecular weight of HA was set to 2×10^4 daltons. The
resulting HA-HZ was dissolved in distilled water, followed
by addition of 1M phosphate buffer (pH 8.8) to prepare
10 0.1 M phosphate buffer having a HA concentration of
50 mg/mL. Methacrylic anhydride was added dropwise in an
amount of 20-fold equivalents relative to HZ and reacted
while stirring overnight at room temperature. After
precipitation in tetrahydrofuran, the precipitate was
15 collected and dried. This precipitate was dissolved in
distilled water, precipitated again in tetrahydrofuran and
then dried. The dried product was then dissolved in
distilled water and lyophilized to give the titled HA-HZ-MA.

[0087] The introduction rate of methacryloyl groups
20 was determined by proton NMR (calculated by comparing
methyl protons in N-acetyl groups (1.8 to 1.9 ppm) for HA
and $\text{CH}_2=$ in methacryloyl groups (5.5 to 6.1 ppm) for MA).
The introduction rate of MA was 22%.

[Example 11]

[Example 11-1] Synthesis of amino (AM) group-modified hyaluronic acid derivative HA-AM

[0088] [Formula 9]



5

[0089] Sodium hyaluronate (HA) having a molecular weight of 2.0×10^5 daltons (Denki Kagaku Kogyo Kabushiki Kaisha, Japan) was converted into a tetrabutylammonium salt form using DOWEX 50WX8-400 (Sigma-Aldrich Corporation) which had been treated with tetrabutylammonium hydroxide (Sigma-Aldrich Corporation).

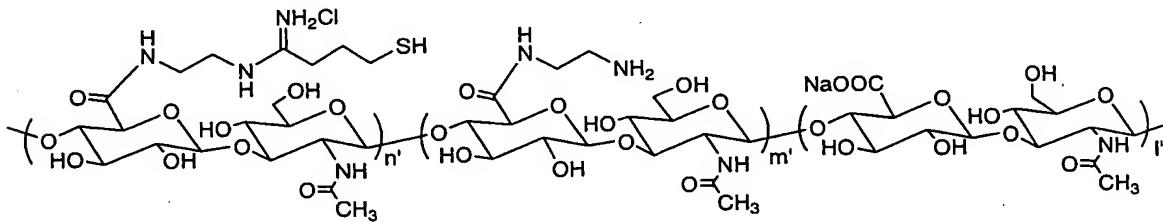
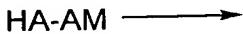
[0090] After the resulting tetrabutylammonium salt of hyaluronate (HA-TBA) was dissolved at a concentration of 2.0 mg/mL in DMSO (Wako Pure Chemical Industries, Ltd., Japan), ethylenediamine (EDA; Sigma-Aldrich Corporation) and BOP (Wako Pure Chemical Industries, Ltd., Japan) were

added in this order at an equivalent ratio of HA unit/BOP/EDA = 1/2.5/50 (mol/mol/mol) and reacted overnight at room temperature. After 1M aqueous sodium chloride was added in an amount of half the volume of the reaction 5 solution, the resulting mixture was reduced to pH 3 with 5N HCl and further neutralized with 2N NaOH. The reaction mixture was dialyzed sequentially against large excess volumes of 0.3 M aqueous sodium chloride and distilled water (SpectraPor 4, molecular weight cutoff (MWCO): 10 12k-14k daltons); ultrafiltered and lyophilized to give the titled amino group-modified hyaluronic acid (HA-AM).

[0091] The introduction rate of amino groups was determined by proton NMR (calculated by comparing methyl 15 protons in N-acetyl groups (1.8 to 1.9 ppm) for HA and methylene protons in the ethylenediamine moiety (2.9 to 3.1 ppm) for AM). The introduction rate was 88.5%.

[Example 11-2] Synthesis of mercapto (SH) group-modified hyaluronic acid derivative HA-AM-SH

[0092] [Formula 10]



HA-AM-SH

20

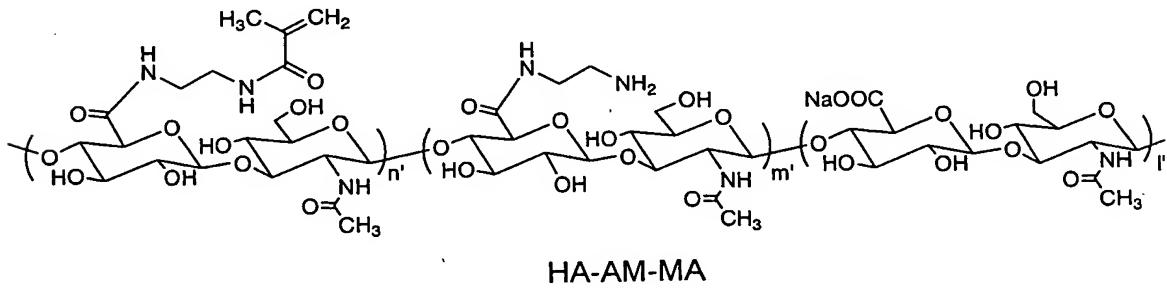
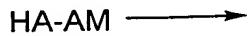
[0093] After HA-AM obtained above was dissolved at 2 mg/mL in carbonate buffer (pH 9), iminothiolane (Pierce)

was added in an amount of 0.5- or 1-fold equivalent relative to HA units and reacted for 45 minutes at room temperature. After the reaction, the reaction mixture was purified on a PD-10 column (Amersham Biosciences) 5 equilibrated with 0.005 N aqueous HCl, and then lyophilized to remove the solvent. The resulting polymer was washed with an excess volume of ethanol and dried under reduced pressure to give HA-AM-SH.

[0094] The introduction rate of mercapto groups was 10 determined by proton NMR in a state containing a reducing agent tris(2-carboxyethylphosphine) hydrochloride (TCEP) (calculated by comparing methyl protons in N-acetyl groups (1.8 to 1.9 ppm) for HA and methylene protons adjacent to mercapto groups (2.4 to 2.7 ppm) for SH). The introduction 15 rate was 16.5% and 23.5% in the respective cases.

[Example 11-3] Synthesis of methacryloyl (MA) group-modified hyaluronic acid derivative HA-AM-MA

[0095] [Formula 11]



20

[0096] After HA-AM obtained above was dissolved at 10 mg/mL in phosphate buffer (pH 7), methacrylic acid activated with 1-ethyl-3-(3-

dimethylaminopropyl)carbodiimide (EDC) was added in an amount of 0.5-, 1.0- or 2.0-fold equivalents relative to HA units and reacted for 2 hours at room temperature. After the reaction, the reaction mixture was dialyzed 5 sequentially against 0.3 M aqueous sodium chloride and distilled water (SpectraPor 4, molecular weight cutoff (MWCO): 12k-14k daltons) for purification purposes, and then lyophilized to give the above polymer.

[0097] The introduction rate of methacryloyl groups 10 was determined by proton NMR (calculated by comparing methyl protons in N-acetyl groups (1.8 to 1.9 ppm) for HA and CH₂= in methacryloyl groups (5.5 to 6.1 ppm) for MA). The introduction rate was 14.8%, 31.9% and 68.9% in the respective cases.

15 [Example 12] Effect of thermal treatment on microparticles (swelling inhibition)

HA-HZ-SH (introduction rate of SH groups: 37.5 mol%) synthesized in Example 9-2 (100 mg) was dissolved in 8.5 mL of distilled water. To this solution, 1 mL of 100 mM phosphate buffer (PB; pH 7) was added and 5 mg of Tween-80 20 was further dissolved, followed by addition of STT (2.3 mg, 1/10-fold molar amount relative to SH groups). This solution was spray-dried under the same conditions as used in Example 1-3 (Solution feed rate 0.5 mL/min, Aspiration speed = 100%) to obtain microparticles. These 25 microparticles were cured in a 50°C thermostat (DN-42, Yamato Scientific Co., Ltd., Japan) and sampled after 24 and 72 hours.

[Test Example 4]

Each sample sampled in Example 12 was measured for particle water content by TGA. The results obtained are shown in Figure 12. Likewise, 30 particles were randomly selected from each sample and measured for their Feret's diameter (dry particle diameter) by microscopic image analysis. Further, the sampled particles were swollen by addition of PBS containing Tween-80 (0.05%) and measured for their wet particle diameter in the same manner. The results obtained are shown in Figure 13. These results confirmed that the degree of swelling was reduced when the particles were incubated for 24 hours. This would be because incubation at 50°C for 24 hours may increase crosslinking within the particles.

[Example 13] Preparation of crosslinked HA-HZ-MA microgels

HA-HZ-MA synthesized in Example 10 (100 mg) was dissolved in 6 mL of distilled water. To this solution, 1 mL of 100 mM phosphate buffer (PB; pH 8.5) containing DTT (11 mg) and TEA (32.5 µL) was added and further mixed with distilled water (3 mL). This solution was spray-dried under the same conditions as used in Example 12 (exhaust temperature 65°C) to obtain microparticles. These microparticles were cured in a 50°C thermostat (DN-42, Yamato Scientific Co., Ltd., Japan) for about 72 hours to obtain particles.

[Test Example 5]

The particles obtained in Example 13 were placed on a slide and, after addition of PBS (pH 7), were then observed

for their state under a microscope, indicating that the particles were not dissolved in PBS. This microscopic observation confirmed crosslinkage formation caused by addition reaction between a mercapto group and an unsaturated bond in the microparticles obtained in Example 13.

INDUSTRIAL APPLICABILITY

[0098] The sustained-release drug carrier of the present invention allows *in situ* chemical crosslinking of drugs such as proteins or peptides and their encapsulation into HA gels while retaining their biological activity, and enables the provision of injectable microparticles which allow long-term sustained release of the drugs such as proteins or peptides at high recovery rates.